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## Formation and regeneration of rhombomere boundaries in the developing chick hindbrain

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### Summary

Development in the chick hindbrain is founded on a segmented pattern. Groups of cells are allocated to particular segmental levels early in development, the cells of each segment (rhombomere) mixing freely with each other, but not with those of adjacent segments. After rhombomere formation, cells in the boundary regions become increasingly specialised. Rhombomeres are thus separate territories that will ultimately pursue different developmental fates. We are investigating the mechanisms that establish and maintain the pattern of rhombomeres and their boundaries. Donor-to-host transplantation experiments were used to confront tissue from different axial levels within the hindbrain. The frequency of boundary regeneration and patterning in the hindbrain was then assessed, based on gross morphology, arrangement of motor neurons and immunohistochemistry. We found that when rhombomeres

from adjacent positions or positions three rhombomeres distant from one another were confronted, a normal boundary was invariably reconstructed. Juxtaposition of rhombomere 5 with 7 also yielded a new boundary. By contrast, donor and host tissue of the same positional origin combined without forming a boundary. The same result was obtained in combinations of rhombomeres 3 and 5. Confrontation of tissue from even-numbered rhombomeres 4 with 6 or 2 with 4 also failed to regenerate a boundary in the majority of cases. These results suggest that cell surface properties vary according to rhombomeric level in the hindbrain, and may support the idea of a two-segment periodicity.

**Key words:** rhombomere, hindbrain, transplantation, chick, cranial nerve.

### Introduction

Early in its development, the vertebrate embryo hindbrain manifests a series of swellings along its length, termed rhombomeres. Despite extensive documentation of rhombomeres in a wide variety of species (reviewed in Keynes and Lumsden, 1990), only recently has the gross morphology been related to an underlying cellular organisation. Studies on the chick embryo shows that neurogenesis has a segmented plan (Lumsden and Keynes, 1989). Differentiated neurons first emerge in rhombomeres 4, 2 and 6, and only later appear in the odd-numbered segments. Cranial nerve roots also bear a strict relationship to the rhombomeric pattern. Retrograde labelling of motor axons of nerve V (trigeminal) at Hamburger and Hamilton stage 16 shows their location in rhombomere 2, with an exit point towards the lateral margin of the brain. One developmental stage later, motor neurons originating in rhombomere 3 (r3) are also labelled, their axons coursing anteriorly to join those from r2. Similarly, motor axons of nerve VII (facial) are situated in r4 and r5, exiting in r4, and those of nerve IX (glossopharyngeal) in r6 and r7, exiting in r6.

Since development of discrete hindbrain structures

(eg. motor nuclei) is segment specific, it is likely that patterning depends on the establishment of rhombomeres as independent territories within the continuous neuroepithelial sheet. This could be accomplished in several ways. In the higher vertebrate embryo, the somitic mesoderm becomes segmented by a process of rostral-to-caudal budding into a series of epithelial somites separated by clefts. Lineage analysis of cells in the segmental plate has shown that labelling of single cells gives rise to clones in which cells have clearly crossed the presumptive intersomitic cleft (Stern *et al.* 1988). This suggests that somites are not developmental 'compartments' in the same way as the insect epidermis. In the integument of *Drosophila*, polyclonal groups of epidermal cells are allocated to anterior and posterior compartments and do not subsequently mix. In the wing, for example, anterior (A) and posterior (P) cells meet along a straight line that is invisible, but can be revealed by cell marking experiments (Garcia-Bellido *et al.* 1973). Thoracic and abdominal segments also contain such a cryptic A/P compartment border, whereas the boundary between adjacent segments (P/A), also a compartment border, is delineated by a change in pigmentation and contour. Thus, whether the compartment border is morphologically recognisable or

not, insect development accomplishes segmentation without recourse to the creation of a physical gap between segments, as in the case of the somites. In this respect, the chick hindbrain more closely resembles the insect example. It is not clear to what extent insect compartment borders present a mechanical impediment to the movement of cells. In *Drosophila*, a zone of non-proliferating cells has been described, coinciding with the interface between the dorsal and ventral lineage compartments of the wing (O'Brochta and Bryant, 1985).

The question therefore arises as to what mechanism is employed during segmentation in the hindbrain. Thus far, the data indicate a parallel with compartment formation in insects. Single cell marking experiments have indeed shown that rhombomeres are polyclonal units of cell lineage restriction (Fraser *et al.* 1990). Although clonally related cells are able to disperse widely and mix amongst clonally unrelated cells within a rhombomere, the progeny of a single cell cannot move across the boundary between two rhombomeres from the time at which the segments first appear. Judged on their conspicuousness, rhombomere boundaries may be similar to the P/A insect segment borders. However, it is interesting that within the hindbrain some boundaries are less morphologically prominent (eg. the r1/2 and r6/7 boundaries) than others, and yet still separate distinct cell lineages.

Rhombomere boundaries possess a number of specialised properties. Boundaries are regions of low cell density, with the majority of cell bodies lying towards the ventricular surface leaving many spaces basally that may be filled with matrix components. Below the boundary ridges are embrasures which, as neurogenesis proceeds, fill with axons. Boundary regions contain the extracellular matrix glycoprotein laminin, and the adult (more highly adhesive) form of N-CAM shows enhanced levels of expression compared with the embryonic (less adhesive) form of N-CAM (Lumsden and Keynes, 1989). Boundaries also contain concentrations of filamentous actin (Guthrie *et al.* 1991), and show enhanced binding of peanut lectin, reflecting a distinct repertoire of glycoproteins (Layer and Alber, 1990).

Pulse labelling with the thymidine analogue bromodeoxyuridine has revealed the acute distribution of S-phase nuclei in the hindbrain. From stage 10, shortly after most boundaries have appeared, up to stage 16, the interkinetic nuclear migration characteristic of the neuroepithelium is reduced or absent at rhombomere boundaries. While in the centres of rhombomeres, labelled nuclei occupy a tight band at the ventral side of the neuroepithelium, at boundaries they approach much closer to the ventricular side (Guthrie *et al.* 1991). The existence in the boundaries of a population of relatively static cells may help maintain the boundary as an impassable zone. As an alternative, or additional mechanism, the provision of immiscibility between cells on either side of boundaries might contain adjacent populations.

What mechanisms are involved in the generation and

maintenance of the periodic pattern in the hindbrain? Rhombomere boundary properties may simply evolve at the interface between cell groups that express different genes, and are already effectively separate. We wanted to investigate the effect of rhombomere identity on the ability to form a boundary. For this, we confronted rhombomeres from different levels within the hindbrain, having removed the boundary cells that might already have established some of the aforementioned properties. The assumption was that donor pieces of hindbrain, transplanted to a novel location in a host, would retain the identity of their place of origin. In this study, therefore, we did not attempt to explore the factors responsible for establishing rhombomere identity. Pieces of tissue were transplanted with part of the underlying mesenchyme still attached. We then examined the tendency of juxtaposed rhombomere cells to make a new boundary, or not, in order to determine whether this property depended on axial position of origin within the hindbrain.

## Materials and methods

Rhode Island Red hens' eggs were incubated to stages 9–12 (Hamburger and Hamilton, 1951), when they were windowed and embryos made visible by sub-blastodermal injection of India ink. Microsurgery was performed through a small opening in the vitelline membrane using needles, flame-sharpened from 100 µm diameter pure tungsten wire.

### Boundary removal experiments

Rhombomere boundaries were removed by aspiration of a 20 µm wide transverse strip of hindbrain, using a micropipette (Fig. 1). In addition to the neuroepithelial cells, some of the underlying mesenchyme was also removed. In order to assess the efficiency of boundary removal and the time course of repair, embryos were removed and fixed immediately after boundary aspiration and at subsequent incubation times ranging from 2 to 24 h. Embryos were then processed for immunohistochemistry.

### Rhombomere transplantation experiments

For transplantation experiments, stage 10–11 embryos were used, with donor and host ages matched as far as possible to within 1 somite stage. Brain pieces were then excised unilaterally, leaving one side undisturbed as a control using transverse cuts at either end, and cutting longitudinally along the floor plate-basal plate boundary (Fig. 2). Some pia mesenchyme was included with the donor brain pieces. Pieces of hindbrain were removed from host embryos in the same manner, leaving the notochord and floor plate in place. Grafts were apposed omitting boundary cells at the junction between donor and host tissue (see Results). Transplants were marked with a spot of carmine at their anterior edge, and inserted into host hindbrains in their normal polarity, using needles.

Operated eggs were sealed with tape and returned to the incubator and high RH for 24–48 h. Embryos were incubated to stages 15–19 and all cases in which grafts had failed to incorporate adequately were excluded from further analysis. Brains in which grafts had healed were either dissected out and mounted flat, processed for histology, or the motor neurons retrogradely labelled using fluorescent dyes. In order to observe final morphology in grafted embryos, hindbrains were dissected free from the embryo, their roof plates opened

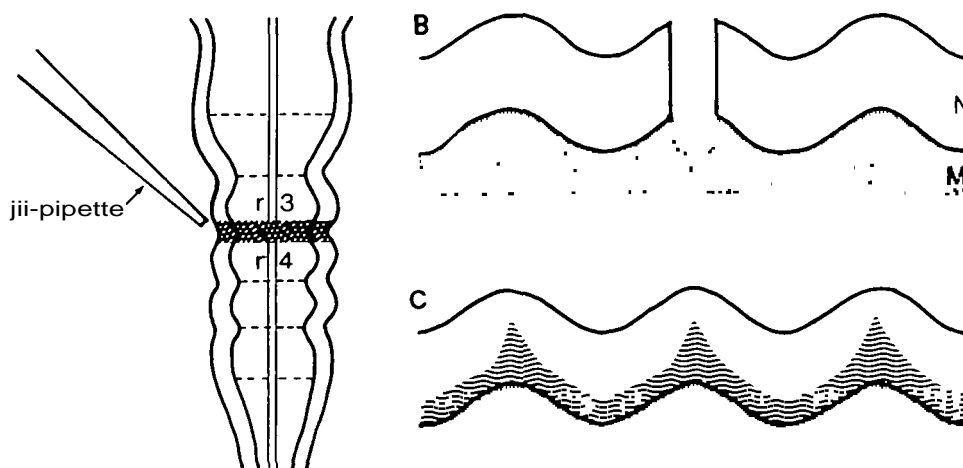


Fig. 1. Diagram of boundary removal experiment. (A) Diagram of stage 11 hindbrain, with dorsal part of neural tube removed. The rhombomere 3/4 boundary region aspirated by micropipette is indicated by the hatched area, and the other boundaries by dotted lines. (B) Schematic parasagittal section through stage 11 hindbrain, showing the region of the neuroepithelium (N) and the mesenchyme (M) removed by aspiration. (C) Schematic parasagittal section through a stage 17 hindbrain, 36 h after

boundary removal, showing the reconstruction of the ablated boundary, and the normal pattern of neurofilament staining (wavy lines) in the marginal layer of the neuroepithelium and extending up into the boundaries.

with a midline longitudinal incision, and then observed from the ventral side as a flattened whole mount using Nomarski optics. The pattern of rhombomeres and boundaries was assessed, comparing the control with the grafted side.

#### Immunohistochemistry

Embryos from boundary removal experiments and transplantation experiments were fixed in 4% paraformaldehyde, embedded in OCT (Miles) and sectioned in the frontal plane at 10  $\mu$ m. In the case of grafted embryos, each section thus displayed both the control and operated sides of the brain. In both boundary ablated and transplanted embryos, immunolocalisation was carried out by indirect biotin-streptavidin immunofluorescence using monoclonal supernatant 3A10, which recognises a 40 kDa neurofilament-associated protein (kind gift of Dr J. Dodd). In boundary ablation studies, a primary antibody against laminin (kind gift of Dr J. Winter) was also used.

For retrograde labelling of cranial nerves, embryos were fixed for 2 h in 4% paraformaldehyde, and solutions of the fluorescent carbocyanine dyes Dil and DiO (Molecular Probes, Oregon, USA, 3 mg/ml in dimethylformamide) were pressure injected into the cranial nerve roots, just lateral to the brain. Labelled embryos were incubated for 24 h at room temperature, in 4% paraformaldehyde (Godement *et al.* 1987). Brains were then dissected free of mesenchyme and mounted flat, ventral side up, and viewed with epifluorescence using green (Dil) or blue (Dil+DiO) excitation.

## Results

#### Description of the microsurgery

The hindbrain neuroepithelium is divided into 8 visible rhombomeres (r1 to r8), whose intervening boundaries (r1/2 to r7/8) are visible to varying degrees at different stages of development. Boundaries appear in a specific sequence, starting with 5/6 at stage 9—, then 3/4 at stage 9, 2/3 at stage 9+, 4/5 at stage 10–, 1/2 at stage 11+, and finally 6/7 at stage 12– (Vaage, 1969; Fig. 1 in Lumsden, 1990). Boundaries delineating rhombomeres 2, 3, 4, 5 and 6 are thus evident by stage 10. For these experiments, it was important that boundaries could be

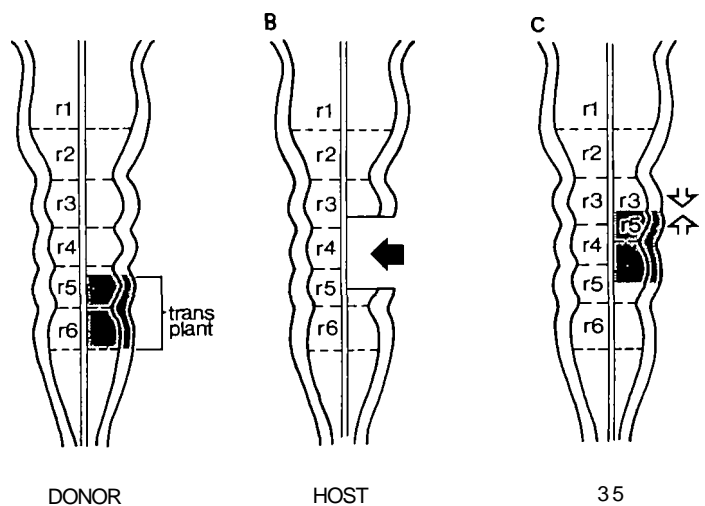


Fig. 2. Diagram of rhombomere transplantation experiments: example of juxtaposition of r3 with r5. (A) Stage 10-11 hindbrain of donor embryo (all rhombomere boundaries drawn in as dotted lines, irrespective of whether they are morphologically apparent at this stage). Shaded area of r5 and r6 is removed, omitting the anterior quarter of r5. (B) Hindbrain of isochronic host embryo, from which r4 and part of r5, plus posterior quarter of r3, has been removed. Donor piece is then inserted, with correct polarity. (C) Host embryo once grafted piece is in place, showing that now r3 and r5 tissue is brought into apposition in the absence of pre-existing boundary cells.

recognised so that boundary cells could either be removed (in boundary ablation experiments), or omitted (in transplantation experiments). Thus, experiments involving boundaries between r2 and r6 were done at stages 10 or 11, while those involving r7 were done at stage 12–.

In transplantation experiments, a unilateral piece of hindbrain about 1.75 rhombomeres in length was removed from the donor embryo (Fig. 2). A piece the

same size was excised from one side of the axis in the host at the desired level. Transplants would thus produce novel appositions of rhombomeric tissue at both ends of the donor piece. However, we restricted ourselves to monitoring events at one end of the graft. In each case, the boundary cells of the rhombomeres that would be confronted were removed, usually equivalent to one quarter of a rhombomere length. For example, in a combination of r3 against r5 (3:5), a donor explant containing most of r5 and all of r6 was placed caudal to a host r3. Here, the anterior boundary of r5 in the donor was omitted, while the posterior boundary of r3 in the host was removed, so that donor r5 confronted host r3 in the absence of boundary cells. The grafted piece retains its normal anteroposterior and dorsoventral polarity. One important difference between this situation and that obtaining in boundary removal experiments is that, in the latter, a space remains while, in transplants, the cut sides abut one another. Attempts were made to align correctly the edges of the neuroepithelium, as this was judged to aid healing and subsequent morphogenesis of the hindbrain.

#### Boundary removal experiments

The removal of a rhombomere boundary was followed by complete reconstruction of the boundary, assessed on morphological and immunohistochemical criteria. Boundary properties are first manifest between stages 9 and 12 as the internal ridges that alternate with the rhombomeric bulges of the neuroepithelium. Based on our present knowledge, boundary characteristics are successively manifested between stages 12 and 17 and possibly even later. Notable is the appearance of a prevalence of adult N-CAM and, as neurogenesis progresses, the accumulation of neurofilament-positive axons, which are concentrated in the pial delta of the boundary, a region of low neuroepithelial cell density. Thus, boundary formation can be seen as progressive. This implies that the reconstruction of a boundary after ablation is a process requiring reiteration of various of the steps of boundary formation, depending on the stage at which it was ablated. We found that, even up until stage 13, boundaries could be rebuilt after ablation, after which time the shape of the hindbrain renders the operation difficult. To examine the time course of reconstruction, however, we performed the operation at stages 10 and 11. A horizontal section through an embryo fixed immediately after removal of the 3/4 boundary shows the interruption in the characteristic laminin staining, which is strong and continuous in the basal lamina abutting the brain, but weaker and punctate in the neuroepithelium (Fig. 3A). In embryos fixed 2 h after the operation, there is a progressive filling of the gap, caused either by buckling of the neuroepithelium to appose the two edges of the wound, and/or proliferation of cells at either edge, and the basal lamina appears to be once more continuous. By 24 h many neurons have differentiated, collecting beneath the reconstructed 3/4 boundary ridge (Fig. 3C)

**Fig. 3.** Reconstruction of the r3/4 boundary following ablation. (A) Horizontal section of a stage 11 hindbrain stained for laminin, immediately following removal by aspiration of the r3/4 boundary. Region removed is shown by arrowheads, where basal lamina is absent and some mesenchyme has also been removed. Scale bar=80  $\mu$ m. (B) Laminin-stained hindbrain 2 h after boundary ablation, showing that cut edges have come together (arrowheads) and basal lamina appears continuous. Scale bar=80  $\mu$ m. (C) Parasagittal section of stage 19 hindbrain 36 h after boundary ablation, showing neurofilament staining in boundaries, including the reconstructed r3/4 (arrowheads) one. Scale bar=100  $\mu$ m.

**Fig. 4.** Formation of new rhombomere boundaries in 3:4 and 3:6 rhombomere graft combinations. (A,D) Schematic diagrams of stage 17 flat-mounted hindbrains resulting from transplantation experiments carried out at stage 10–11; 3:4 and 3:6 grafts respectively. The grafted tissue is stippled and regenerated boundary indicated by an arrow and heavy shading. (B,E) Examples of flat-mounted brains from stage 17 3:4 and 3:6 grafts, respectively, photographed with Nomarski optics. Boundaries appear as darker regions, and rhombomeres are numbered. The anteroposterior extent of the graft is indicated by a solid line, and the regenerated boundary by black arrowheads. (E) The r6/7 boundary in the grafted tissue is not prominent. Scale bar=110  $\mu$ m. (C,F) Horizontal sections of hindbrains in 3:4 (stage 15) and 3:6 (stage 17) grafted embryos, stained for neurofilament-associated proteins. White arrowheads indicate regenerated boundaries. (C) r4 shows the most intense staining. (F) Posterior to the r3:6 boundary on the operated side, the r6/7 boundary is not prominent. Scale bar=85  $\mu$ m.

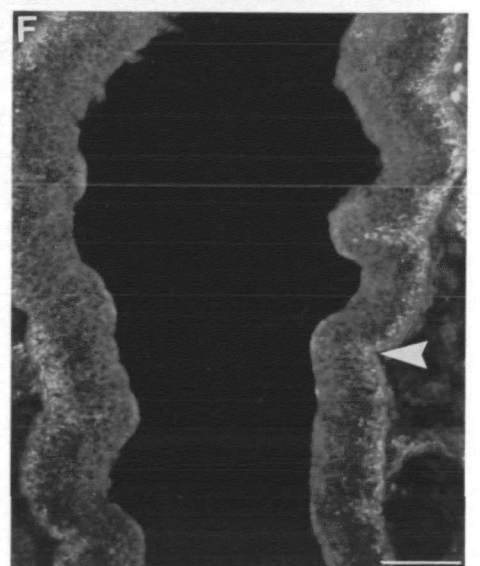
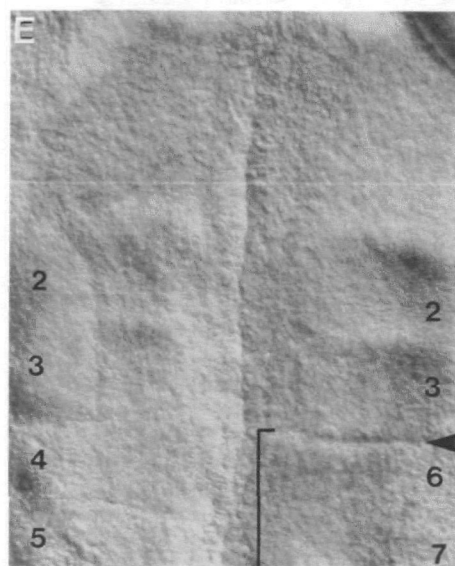
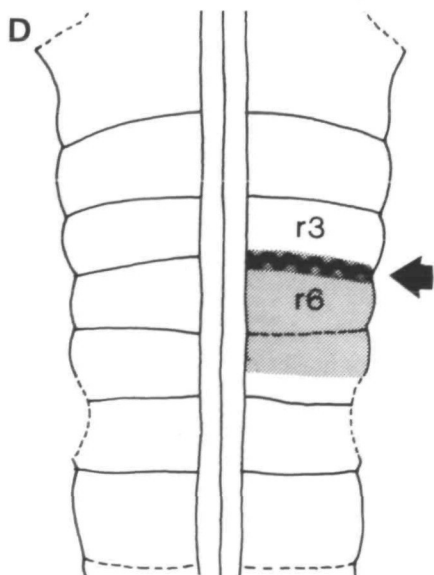
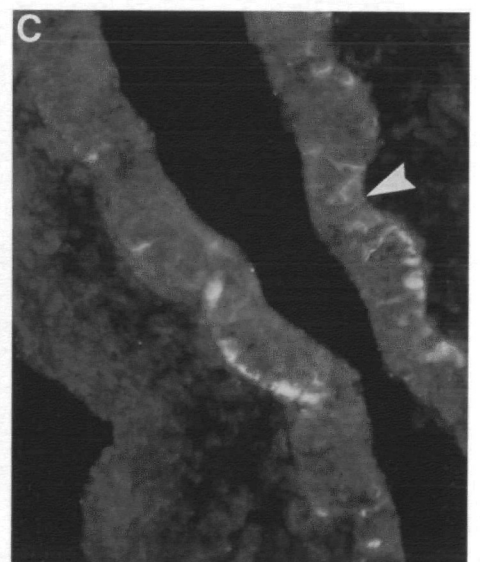
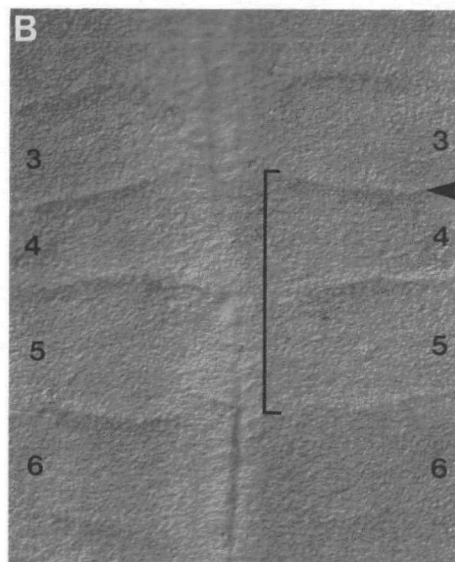
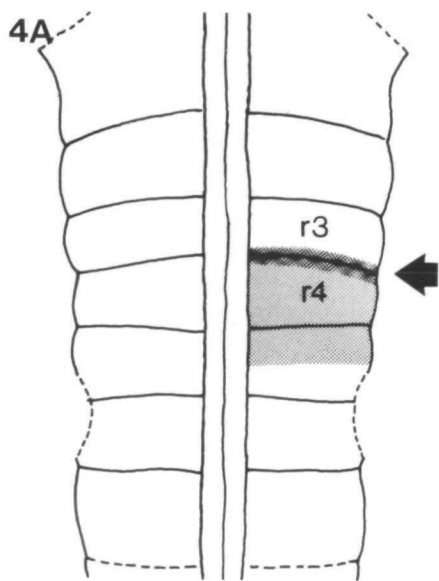
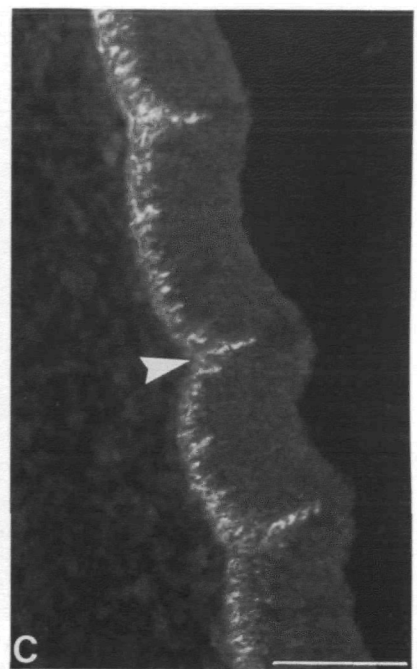
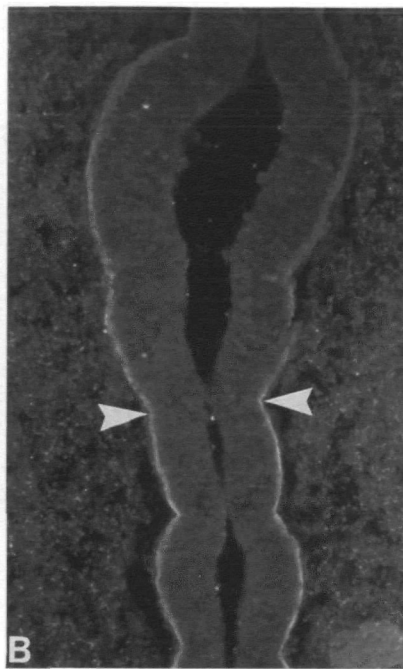
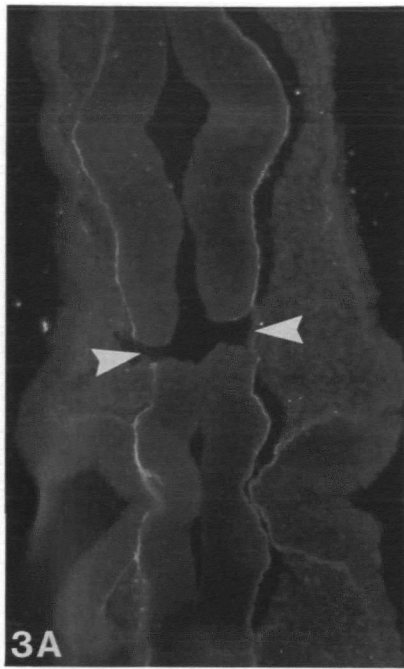
which has the same morphological appearance as the neighbouring boundaries.

#### Transplantation experiments

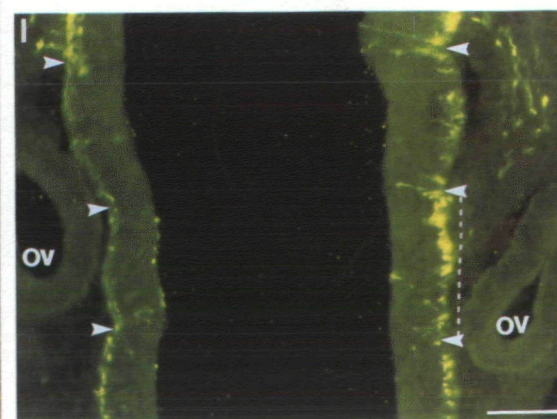
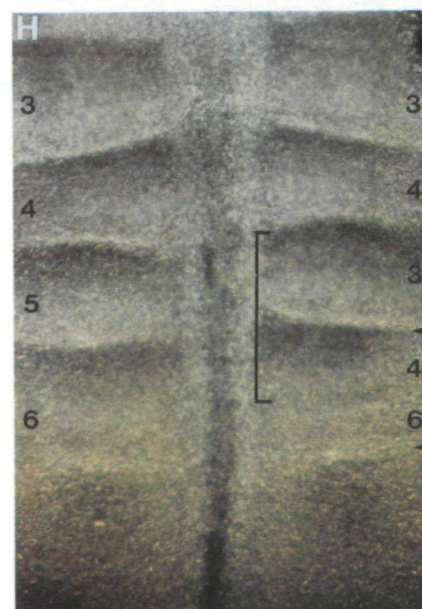
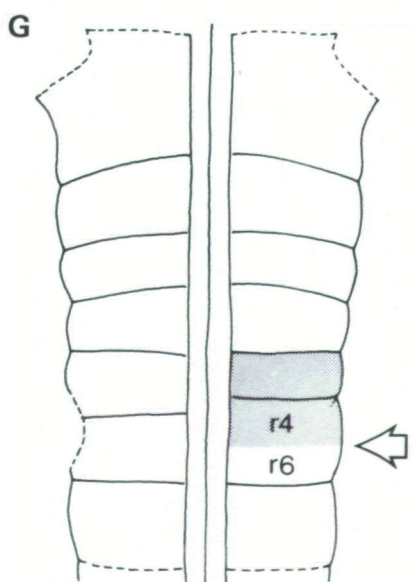
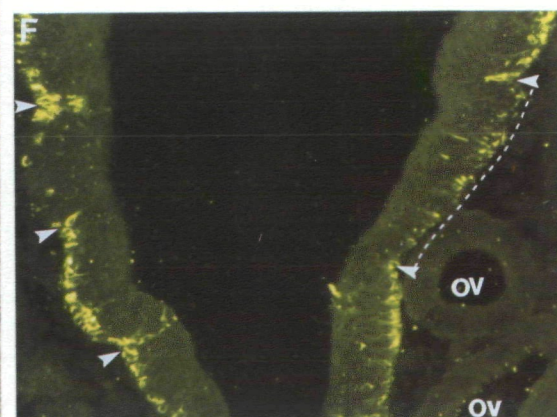
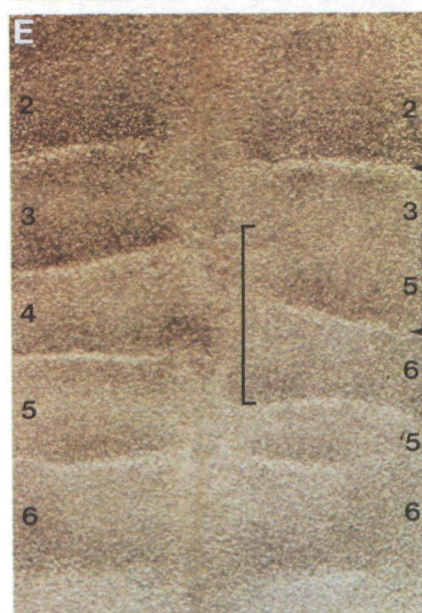
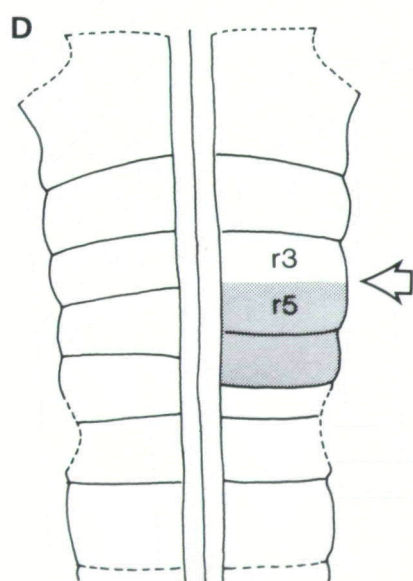
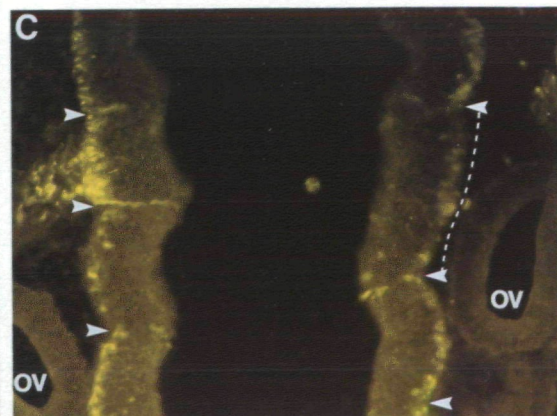
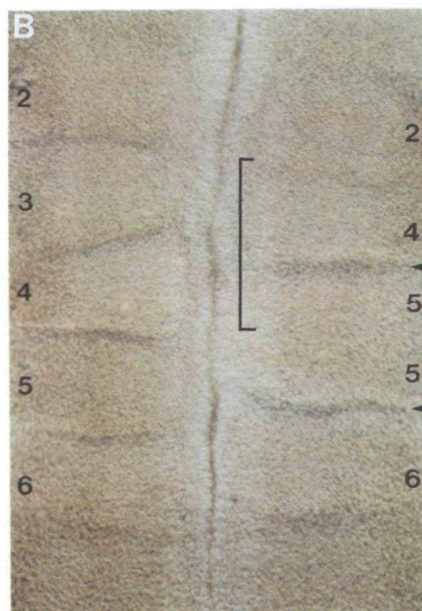
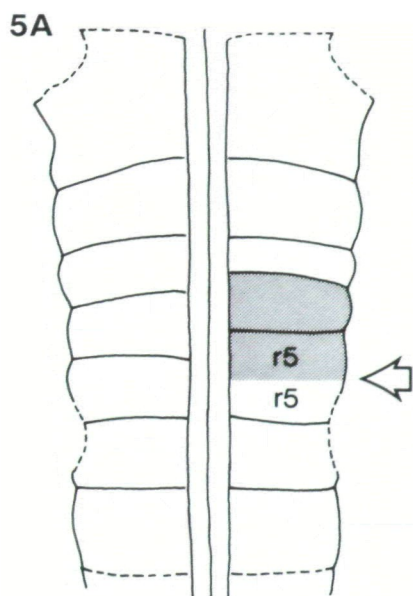
Only about 33% of transplanted embryos healed adequately to be assessed. The formation of a new boundary was easily distinguished from inadequate healing, which was marked by deformation of the neuroepithelium, and intrusion of basal lamina and/or mesenchyme between the two edges. In all transplantation experiments, grafted brain pieces retained their phenotype in their new location, as will be discussed. The frequency of formation of new boundaries in transplanted embryos is described below and in Table 1. Figs 4–6 show grafted hindbrains in which the control side is always on the left and the grafted side on the right.

#### Controls: r3:4 and r4:5 grafts

To discover whether boundaries could be rebuilt in grafts in the same manner as in boundary removal experiments, r3 was confronted with r4 in the absence of the r3/4 boundary. In both r3:4 and r4:5 grafts, the donor rhombomere was placed posterior to the host rhombomere, eg. donor r4 placed posterior to host r3. In r3:4 grafts, a new boundary formed ( $n=8$ ) and the operated side was virtually indistinguishable from the unoperated one. These results are shown in Table 1. In Fig. 4B, a stage 17 hindbrain flat-mounted and viewed

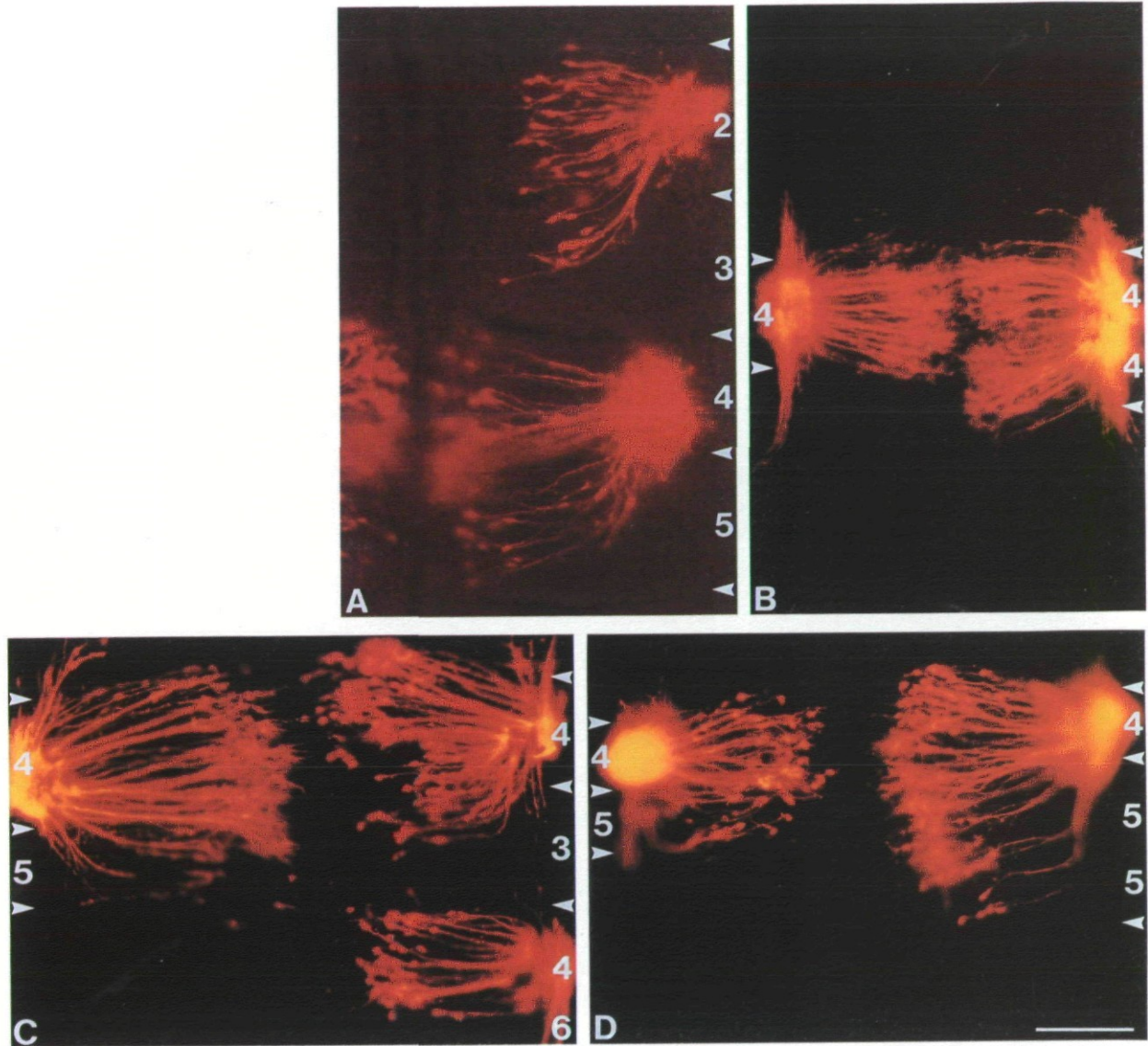






**Fig. 5.** Absence of rhombomere boundaries in 5:5, 3:5 and 4:6 rhombomere graft combinations. (A,D,G) Schematic diagrams of stage 17 flat-mounted hindbrains resulting from 5:5, 3:5 and 4:6 grafts, respectively. The grafted tissue is stippled, and the absence of a boundary at the interface between host and graft is shown by the open arrow. (B,E,H) Examples of flat-mounted brains from stage 17, 5:5, 3:5 and 4:6 grafts respectively, photographed with Nomarski optics, with rhombomeres numbered. Boundaries appear as darker regions (B) or lighter regions (E,H). The anteroposterior extent of the graft is indicated by a solid line, and the enlarged rhombomere resulting from the juxtaposition of host and graft tissue by a dotted line with arrowheads at either end. Scale bar=110  $\mu$ m. (C,F,I) Horizontal sections of stage 17 hindbrains in 5:5, 3:5 and 4:6 grafts, respectively, stained for neurofilament-associated proteins. White arrowheads indicate rhombomere boundaries, and the dotted line indicates the enlarged rhombomere composed of host and donor tissue, which does not contain a boundary. (C) On the control (left) side, r4 is evident by the intense staining, and the presence of the facial ganglion lateral to the brain. The otic vesicle (OV) is located between r5 and r6 on both sides. (F) On the control side, r2 and r4 are distinguished by the enhanced neurofilament staining, since at this stage neurogenesis is advanced in the even rhombomeres. On the operated side, there are two otic vesicles, the anterior of which (at the posterior end of the enlarged rhombomere) is derived from remnants of the otic placode attached to the grafted piece. (I) On the operated side, the otic vesicle has been shifted posteriorly during grafting and lies at the 6/7 boundary, which is poorly defined. The grafted r4 is distinguished by the abundance of neurofilament staining. Scale bar=85  $\mu$ m.





**Fig. 6.** Retrograde DiI labelling of motor neurons in normal and grafted hindbrains. (A) One side of normal stage 17 hindbrain, phase contrast and epifluorescence showing labelled motor neurons of the trigeminal nucleus (in r2 and r3) and the facial nucleus (in r4 and r5). Note distinctive fan-shaped array of facial neurons with cell bodies near the midline. Note also that neurons in odd-numbered segments arc anteriorly to exit in r2 and r4. Scale bar=110  $\mu$ m. (B) 4:4 graft showing normal facial nucleus on the control side, and reduplicated facial nuclei in the host and graft r4s on the operated side, both with normal morphologies and a common exit point. Scale bar=160  $\mu$ m. (C) 4:6 graft showing (right side) an extra facial nucleus, posterior to the host facial nucleus, lying in the anterior part of the new r4-6 and with its own exit point. Scale bar=130  $\mu$ m. (D) 5:5 graft. On the control (side), the facial nucleus is shown occupying r4 and r5. On the operated side, an r5 graft has been placed posterior to the host r5 and motor axons from the grafted 5 can be seen curving anteriorly to join those of the host r5 and exit in the host r4. Scale bar=160  $\mu$ m.

**Table 1.** Incidence of boundary formation in rhombomere transplantation experiments

Graft combination	Result		
	No boundary	Partial boundary	New boundary
3 4	—	—	8
4 5	—	—	7
3 6	—	—	8
2 5	—	—	8
4 4	7	—	—
5 5	8	—	—
2 4	9	1	2
4 6	15	2	2
3 5	19	—	—
5 7	—	—	10

The left-hand column shows the different rhombomere combinations used in grafting experiments e.g. 3 4 – juxtaposition of rhombomere 3 with rhombomere 4. Graft combinations are paired in five different categories, adjacent rhombomeres, intervals of three rhombomeres, reduplicates, even-numbered and odd-numbered rhombomere combinations. The number of grafted brains showing a particular result are denoted in the right-hand columns as no boundary (failure to form a new boundary), partial boundary (see text), or a formation of a complete new boundary.

with Nomarski optics shows no difference in morphology compared with a normal hindbrain. Similar results were obtained when r4 was juxtaposed with r5 ( $n=7$ ); when such a hindbrain was sectioned, the reconstructed 4/5 boundary is normal, judged by its contour and by immunostaining of boundary axons (Fig. 4C).

#### Three rhombomere intervals: r3:6 and r2:5

In these grafts, a donor piece containing r6 or r5 was placed posterior to the host r3 or r2, respectively. These permutations gave the unequivocal result that a new boundary was formed ( $n=8$  for each graft combinations), the same result as when rhombomeres from adjacent positional levels were confronted. The result was the same irrespective of whether 2:5 or 3:6 combinations were used. Fig. 4 shows a flat-mounted 3:6 embryo with a new boundary (Fig. 4E), and neuronal boundary staining in a similar embryo (Fig. 4F).

#### Reduplicates: r4:4 and r5:5

Two rhombomeres from identical positional levels, rhombomere 5 with 5, were placed in apposition by inserting the donor r5 anterior to the host 5 in the absence of a boundary (Fig. 5A). Examination of whole-mounted brains 24–36 h later showed that the two had united to form a single oversized rhombomere, without generating an intervening boundary ( $n=8$ ) (Fig. 5B). Neurofilament staining revealed that no cryptic boundary had formed in the region where host and grafted tissues met (Fig. 5C). R4:4 grafts, (placing donor r4 either anterior or posterior to the host r4), also failed to produce a new boundary in all cases ( $n=7$ ).

#### Odd-numbered rhombomere combinations: r3:5 and r5:7 grafts

Juxtaposition of r5 with r3 (donor r5 placed posterior to host r3) also resulted in the formation of a compound rhombomere, devoid of a donor-host boundary ( $n=19$ ). Usually this segment was enlarged relative to the contralateral control r3 (Fig. 5E). Sometimes, where the host and grafted rhombomere components both approximated to half a segment in length, the resulting segment was normal sized. A frozen section of such an embryo stained with antibodies against neurofilament-associated protein shows the extent of the compound rhombomere in comparison to the control side (Fig. 5F). Combinations of r5 with r7, placing the donor r7 caudal to the host r5), however, gave a quite different result. In all 10 cases, a new boundary was formed, in the same manner as in grafts shown in Fig. 4.

#### Even-numbered rhombomere combinations: r4:6 and r2:4 grafts

The even-numbered rhombomere combination 4:6 was achieved in two ways; either by placing a donor r4 anterior to the host r6, or by placing a donor r6 posterior to the host r4. There were no consistent differences in the results of the two types of graft. By far the majority ( $n=15/19$ ) showed a final pattern with a compound r4:6, devoid of boundary (Fig. 5H). However, there were also a few examples in which a partial ( $n=2/19$ ) or an entire boundary ( $n=2/19$ ) was formed. In horizontal sections stained to reveal the arrangement of boundary axons, the assessment of brains in which a boundary was judged to be absent was confirmed (Fig. 5I). However, in those judged to contain a partial or entire boundary, there was some variation in the contour of the boundary ridge, and in the abundance of boundary axons.

Rhombomere 2:4 (donor r4 placed posterior to host r2) combinations gave similar results. Again, the majority ( $n=9/12$ ) failed to give rise to a new boundary.

#### Disposition of motor axons in grafted hindbrains

At stage 17, motor neurons have a characteristic distribution in the chick hindbrain (Lumsden and Keynes, 1989). Retrograde dye labelling of cranial nerve roots shows that the neurons of the trigeminal nucleus are located in r2 and r3, exiting from r2, while those of the facial nucleus, lying in r4 and r5, exit from r4. In addition, the trigeminal and facial nuclei have quite distinct morphologies; facial neurons constitute a fan-shaped array with their cell bodies much closer to the midline than the trigeminal neurons. Such a normal pattern is shown in Fig. 6A. Dye labelling of cranial nerves was carried out in grafted embryos to examine the patterning of motor neurons in grafted regions of hindbrain.

The conclusions from such labelling experiments were that motor neurons invariably retained the character of their position of origin. For example, in r4:4 grafts, labelling of the VIIth nerve, whose axons normally emerge from r4, showed a duplication of the typical morphology of the facial nucleus (Fig. 6B). In

some cases, axons coursed through a common exit point in the enlarged r4, while in other cases, nerve VII bifurcated to two exit points, originating in the donor and host tissue, respectively. In combinations of two even-numbered rhombomeres, the same preservation of phenotype was observed. In a compound r4:6, a characteristic fan-shaped array of 'facial' type axons, with their cell bodies close to the midline, was evident occupying the anterior, donor portion of the compound rhombomere (Fig. 6C). Since the grafted tissue contained its own exit point, these axons coursed laterally to exit in r4. Similarly, the axons of the host r6 maintained their normal arrangement and exited in r6. In these experiments, axon fasciculation behaviour outside the brain and navigation to targets seemed to be governed largely by proximity. Although we cannot locate the junction between host and grafted tissue unequivocally without the aid of marking techniques, it seemed to us that in compound r4:6 grafts, motor axons always stayed within their rhombomere of origin. This may not be surprising since branchiomotor exit points are (with the exception of nerves X and XI in r7) located in even-numbered rhombomeres. Exit-points may be specified at least partially by the neural crest (Moody and Heaton, 1983), some of which is transplanted along with the brain during grafting experiments.

In r5:5 combinations, motor axons of the VIIth nerve were distributed throughout r4 and the compound rhombomere 5:5, with neurons from donor and host territories converging on the exit point in r4 (Fig. 6D). This gave rise to a pattern closely similar to that in normal development, albeit that r5 was enlarged relative to normal. R3:5 grafts showed similar axonal behaviour, with motor axons originating from the donor r5 running anteriorly across the host r3 territory to exit in the host r2. In this case r5 axons exited inappropriately, since in normal development they exit in r4. However, the anterior direction of growth and arcuate trajectories of the donor r5 axons suggested that many of their normal characteristics were maintained in the new location. Thus, the motor axons of both even- and odd-numbered grafted rhombomeres retain normal projection patterns within the hindbrain, though axons from odd-numbered rhombomeres sometimes exited in a rhombomere of inappropriate identity (though appropriate position). The proximity of an exit point thus appeared to be an important factor in governing the trajectory of axons.

## Discussion

During development, segmentation of the hindbrain divides it into a series of apparently similar units. Individual rhombomeres thus develop a degree of independence, allowing cells at different rhombomeric levels to pursue different fates. We have investigated the tendency to form new boundaries when cells from different rhombomeric levels were juxtaposed. The results suggest that differences in cell surface properties may be an early manifestation of rhombomere identity

in the developing hindbrain, and may be involved in restricting cell movement between rhombomeres. Available evidence suggests that rhombomere boundaries represent specialised domains. From their earliest appearance, boundaries are regions where cells have reduced rates of division and the typical interkinetic nuclear migration is reduced (Guthrie *et al.* 1991). Boundaries are characterised by low cell density, specific cytoarchitecture and a distinct repertoire and distribution of cell adhesion and substratum adhesion molecules (Lumsden and Keynes, 1989). A detailed analysis of the development of these features might help explain why it is that, once neurons have differentiated and migrated out into the mantle zone, their growth cones are not subject to the same restriction that prevents neuroepithelial cells from crossing boundaries. Possibly, the mixing of neuroepithelial cells is maximally constrained at the apical side, where the greatest opportunity for lateral movement takes place after cell division. Axonal navigation, on the other hand, takes place adjacent to the basal side of the epithelium.

A lineage analysis in the chick hindbrain has recently shown that cells do not move between rhombomeres from the time of first morphological appearance of the rhombomere boundaries (Fraser *et al.* 1990). The role of the boundary in restricting cell movement was explored firstly in boundary removal experiments. Invariably, this resulted in reconstruction of the boundary and development of normal morphology and colonisation by axons. This argues that the boundary does not merely comprise a physical barrier, in the absence of which cells from adjacent levels associate seamlessly. Rather, the encounter of cells from adjacent rhombomeres must entail some recognition of cell surface differences that leads to recreation of the missing boundary. The sequence of events culminating in restoration of the boundary has not been characterised. The space left by boundary removal may trigger cell proliferation on either side, and/or migration of cells to fill any remaining gap. Cells from adjacent rhombomeres may have distinct adhesive properties so that when cells from adjacent territories meet, cells then sort out to form a straight border, forming a new barrier to cell movement. In most experiments, rhombomeres on either side of the ablated boundary were normal in size and morphology. Similarly, removal of a transverse boundary-wide strip of cells from the middle of a rhombomere resulted in regeneration of a segment of normal size (data not shown). Removal of a transverse section of a rhombomere more than three quarters of its anteroposterior extent can result in production of a smaller rhombomere. However, it appears that rhombomere size can undergo efficient regulation after ablation either at or between the segment boundary.

Boundary reconstruction was also elicited by the juxtaposition of cells from adjacent rhombomeres in donor-to-host grafting experiments. Based on motor neuron patterns, rhombomeric tissue was found to retain the phenotype characteristic of its level of origin.

Motor neuron patterning and axon trajectories in grafts appeared to reflect the identity of the grafted rhombomeres; these issues will be addressed in more detail in a subsequent paper. When transplants were carried out at stage 10–11 with the presence of pial mesenchyme, there was no indication that the grafted tissue conformed to its new position. This observation is broadly consistent with data showing that hindbrain r1 alar plate, destined to become cerebellum, maintains its specific character when grafted into the midbrain (Alvarado-Mallart *et al.* 1990). The same authors, however, showed that the gradient of expression of the chick-En gene product adjusted when the portion of midbrain/hindbrain normally expressing it was reversed (Martinez and Alvarado-Mallart, 1990). Though few level-specific markers are yet available for the hindbrain, other indicators such as the characteristic arrangement of motor axons could be assessed. Our conclusion that grafts retain their original character is reinforced by the observation that the same region of the host axis receiving transplants of different donor origin showed different behaviour in the frequency of boundary regeneration.

Boundaries were not rebuilt in all transplants that confronted tissue from rhombomeres with different positional identities. Juxtapositions of adjacent rhombomeres, for example r3:4, or of rhombomeres normally lying three segments distant from each other, for example r3:6, or of rhombomeres 5:7 gave rise to new boundaries in all cases. Combinations of tissue from the same origin, eg. r5:5, or of odd-numbered rhombomeres r3:5 never formed a new boundary. Even-numbered combinations, eg. r4:6 did not form a boundary in a majority of cases. These results may indicate cell surface properties that alternate down the neuraxis, so that odd-numbered rhombomeres 3 and 5 would share a property not held in common with even-numbered rhombomeres 2, 4 and 6. This possibility has theoretical appeal since it would be a simple way of generating the segmented pattern of the hindbrain. Fields of cells could be set aside early in development expressing one or other cell surface property. At the meeting point between two groups of cells with different properties, a boundary would then be established, *via* the specification of a third cell state. That such a hypothesis cannot include rhombomere 7 will be discussed.

The observation of heterogeneous results in even-numbered grafts requires additional interpretation. Observation of morphology and neurofilament staining were the two criteria of boundary formation selected, while use of a broader range of criteria might simplify interpretation. In addition, since rhombomere boundaries appear and mature at different times (Vaage, 1969), differences in the maturation state of particular rhombomeres may account for their slightly different behaviours in graft combinations. The least likely explanation is that there might be continuous changes in positional information within a single rhombomere. In grafts, the intention was to omit tissue equivalent to the posterior quarter of the donor rhombomere and the anterior quarter of the host rhombomere, or *vice versa*,

depending on the graft. However, at early stages reliably removing a known fraction of a particular rhombomere was difficult. As will be discussed, embryonic insect tissue shows a tendency for boundary formation depending on the positional level within the segment of the regions juxtaposed. Therefore, we cannot entirely dismiss fine variation in positional level as a contributory factor to heterogeneity of results.

The observation that juxtaposition of 5 with 7 invariably produced a boundary is unlikely to be explained by the factors described above. One possible interpretation is that r7 does not conform to the pattern of the rest of the hindbrain. Cell lineage analysis has yet to be performed for r7, and the r6/7 and r7/8 boundaries are less prominent than the others. The observation of behaviour at the r5/7 interface may preclude the construction of simple hypotheses to describe complex situations. Rather than postulating discrete differences of odd- and even-numbered rhombomeres, the data may be consistent with a group of cell surface properties, with each even-numbered rhombomere expressing a subset of these, endowing cells of different rhombomeres with the potential to adhere more or less strongly to cells from other locations. This scheme might also be compatible with the idea that only one of each rhombomere pair, odd and even, confers boundary-forming ability on the other during development.

Our observations on boundary reconstruction can be broadly compared with previous work on regeneration of the segment boundary in insects. The insect cuticle is a system that exemplifies the concept of positional information, elaborated and refined by Wolpert (1971). The cuticle of some insects bears structures that are indices of the underlying positional values. Early experimental perturbations showed that patterning of scales or ribs in *Galleria* (Piepho, 1955; Stumpf, 1966) and ripples in *Rhodnius* (Locke, 1959) oriented as though lying in the field of some diffusible substance. In *Oncopeltus*, the segment boundary may be interpreted as separating the source of one concentration gradient from the sink of the next. Moreover, the boundary divides cells of two polyclonal compartments, in the same way as in the chick embryo hindbrain (Fraser *et al.* 1990). Wright and Lawrence (1981) showed that burning or extirpation of the segment boundary resulted in regeneration of a new boundary. Boundary regeneration was thus assumed to arise due to the apposition of cells with disparate positional values. By contrast, removal of an entire segment length from halfway down one segment to halfway down the next did not lead to regeneration of extra tissue, or to the formation of a new boundary, since by this manipulation appropriate positional values were juxtaposed. In some cases, boundary removal did not result in boundary regeneration, but instead, intercalation of positional values within the segment. Other authors (Campbell and Shelton, 1987), maintained that the only means by which boundary regeneration could be explained was that a specialised population of border cells acted to isolate adjacent populations.

Boundary regeneration in the chick hindbrain paralleled that in *Oncopeltus*, since a new boundary between adjacent segments could be formed in the absence of boundary cells. In general, however, our results are more indicative of discrete differences in cell surface properties than a continuous system of positional information. Tendency to form or not to form a boundary depended on the rhombomeric origin of apposing cells rather than the level within the rhombomere. The nature of positional information in the developing chick hindbrain, possibly embodied in a diffusible signal, remains unexplored. In *Oncopeltus*, there is evidence to show that transfer of the small dye Lucifer Yellow *via* gap junctions is more extensive within the segment than across the segment border (Warner and Lawrence, 1982). In *Tenebrio*, the segment border is marked by a strip of particularly impermeable cells (Blennerhassett and Caveney, 1984).

The selective adhesion of cells at different rhombomere levels may provide an explanation for our observations. Disaggregated cells of the germ layers of the amphibian embryo have been shown to reaggregate selectively (Townes and Holtfreter, 1955). The logical extension of the present work is therefore to examine the potential for mixing between cells from different rhombomeres, *in vitro* and *in vivo*. We would predict that cells from adjacent rhombomeres, those three distant from each other, or 5:7 combinations would be immiscible, while those from the same rhombomere, 3:5, 2:4 or 4:6 combinations would be freely miscible. Two pieces of evidence support this idea. First, in the normal chick hindbrain, labelled neuroepithelial cells descended from a single marked precursor can mingle freely with unlabelled, clonally unrelated cells within the rhombomere, but do not move across segment boundaries (Fraser *et al.* 1990). Second, preliminary observations (S. Fraser, R. Keynes and A. Lumsden, unpublished observations) suggest that marking cells on one edge of the gap, immediately after boundary ablation, results in spread of marked progeny along, but never across the regenerated segment boundary. Experiments on boundary formation between cells of two different colour phenotypes in *Oncopeltus* (Wright and Lawrence, 1981) showed a similar phenomenon. In a subsequent study, we will address the question of cell mixing using fluorescent cell marking and chick-quail chimaeras.

A periodicity of cell surface properties in the chick hindbrain may stem from periodic expression of genes significant in development. Various tantalising patterns of gene expression have been described in the early hindbrain, both in the mouse and now in the chick (Sundin and Eichele, 1990). Some genes expressed in the hindbrain are homologous to those expressed early in *Drosophila* embryogenesis. In *Drosophila*, the segmented pattern of the embryo is progressively refined by steps, depending on a series of genes, including ultimately pair-rule genes, and homeotic (Hom) genes (reviewed in Ingham, 1988). Segment identity appears to be defined by subsets of genes, acting in a combinatorial manner (Lewis, 1978). The

same may be true of the hindbrain. In the mouse, genes of the Hox 2 cluster are expressed in the spinal cord and hindbrain (Duboule and Dolle, 1989; Graham *et al.* 1989) and have anterior cut-off points that coincide with rhombomere boundaries (Wilkinson *et al.* 1989b). In the mouse, *Hox2.9* is expressed in rhombomere 4 (Murphy *et al.* 1989), while a homologue of this gene in the chick, *Ghox-lab*, is expressed in r4 and also from r7 caudal, at rhombomeric stages (Sundin and Eichele, 1990). The zinc-finger gene *Krox 20* is expressed in rhombomeres 3 and 5 in both mouse (Wilkinson *et al.* 1989a) and chick (A. Nieto and D. G. Wilkinson, personal communication), having an obvious parallel with the apparent affinity of r3 and r5 cells described by us. The formation of r5/7 boundaries is consistent with the idea that *Krox 20* expression may be somehow linked to cell surface properties. The downstream targets of these genes, all of which code for transcription factors, have not yet been defined. Recently, however, an interesting pattern of glycoprotein distribution has been found in the hindbrain. The HNK-1 epitope, a sulphated carbohydrate structure associated with various adhesion glycoproteins (including N-CAM and L1), is highly enriched on neuroepithelial cells of rhombomeres 3 and 5 relative to the even-numbered segments at stages 14–15 (S. Kuratani, personal communication). We can now anticipate the discovery of more such differences in cell surface properties at different rhombomere levels in the hindbrain.

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